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Photoactive chelates for radiolabelling proteins

Larissa S. Eichenberger,[a] Malay Patra[a,Δ] and Jason P. Holland*[a]

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Photochemical reactions are an attractive foundation for the synthesis of radiolabelled antibodies, immunoglobulin fragments and other proteins/peptides. The synthesis, ^{68}Ga-radiochemistry and photochemical reactivity of three macrocyclic chelates functionalised with an arylazide group is described. Experiments with trastuzumab confirm that photoradiochemistry facilitates fast, one-pot synthesis of radiolabelled proteins.

Standard methods for radiolabelling biologically active proteins or peptides with radionuclides like ^{64}Cu, ^{68}Ga, ^{89}Zr, ^{90}Y, ^{111}In, ^{177}Lu and ^{225}Ac etc, rely almost exclusively on thermochemical processes.[1] For instance, the synthesis of radiolabelled monoclonal antibodies (mAbs) for use in immuno-positron emission tomography (immuno-PET) or radioimmunotherapy (RIT) usually requires pre-functionalisation of the protein with a metal ion binding chelate, followed by radiolabelling in the final step. Many methods exist for coupling the chelate to the protein.[2] These include the use of 'activated' reagents based on esters, maleimido groups or *para*-substituted benzyl-isothiocyanates that facilitate coupling to reactive lysine or cysteine residues *via* amide or thioester bond formation. Bioorthogonal approaches that utilise 'click' chemistry, or site-specific enzymatic ligation of the glycoprotein have also shown promise.[3,4] However, the conjugation kinetics and overall efficiency of each of these methods is ultimately limited by thermochemistry.[5] Heating reactions can often increase the rates of the conjugation or radiolabelling steps but for most proteins, thermal stability is a key concern.

Photochemistry harbours a diverse array of reactions that are useful in the synthesis of organic compounds.[6] However, only a handful of examples exist where photochemistry has been used in the synthesis of radiolabelled compounds.[7–17] We recently demonstrated that a one-pot, photoradiochemical process involving ^{68}Ga-radiolabelling of a macrocyclic chelate and photochemical conjugation with proteins *in situ* facilitates the direct synthesis, purification and formulation of ^{68}Ga-labelled mAbs in <20 min.[18] In efforts to expand the radiochemical scope of this photoradiochemical approach, here we report the synthesis, characterisation and ^{68}Ga-radiochemistry of three new photoreactive chelates derived from the *aza*-macrocycles NOTA, DOTA and DOTAGA. These chelates offer different numbers of donor groups, cavity sizes, and overall charges that can be tuned for coordination of radioactive metal ions (with formal oxidation states of +2, +3, +4 or +5) from across *p*-, *d*- and *f*-blocks.[19]

The photoactive chelates, NOTA-PEG$_3$-ArN$_3$ (**1**), DOTA-PEG$_4$-ArN$_3$ (**3**) and DOTA-PEG$_4$-ArN$_3$ (**4**) were synthesised *via* standard chemical transformations starting from 4-azidobenzoic acid and commercially available reagents (Figure 1). Full experimental details and characterisation data including high-resolution electrospray ionisation mass spectrometry, ultrahigh-performance liquid chromatography (UHPLC), ^{1}H and ^{13}C NMR spectroscopy are presented in the Supporting Information. In all cases, semi-preparative HPLC was used to isolate the compounds in high purity. Compound **1** was synthesised in 37% yield after the *N*-hydroxysuccinimide activated ester (NOTA-NHS) was reacted with a pre-synthesised[18] polyethylene glycol (PEG)-functionalised ArN$_3$ reagent (N$_3$-PEG$_3$-NH$_2$, Figure 2 [green trace] and Supporting Information Figures S1 – S6). Compound **3** was synthesised in 89% yield *via* the reaction of DOTA-PEG$_4$-NH$_2$ with the activated NHS ester, 2,5-dioxopyrrolidin-1-yl-4-azidobenzoate (**2**, Figures S7 – S14). Compound **4** was produced in 29% after direct coupling of DOTAGA-PEG$_4$-NH$_2$ with 4-azidobenzoic acid in the presence of HATU/DIPEA in DMF (Figures S15 – S18). Chromatographic data from experiments using compound **1** are presented in the main text. Equivalent data sets for compounds **3** and **4** are given in the Supporting Information. Note that the PEG linkers were introduced to increase the space between the chelate and the

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photoactivatable ArN_3 group. PEG groups also have the additional benefit of increasing water solubility which is a limiting factor for some chelates.[1] However, it is conceivable that shorter linkers or even direct coupling of ArN_3 to one of the carboxylate arms of the chelates would also generate viable photoactive reagents.

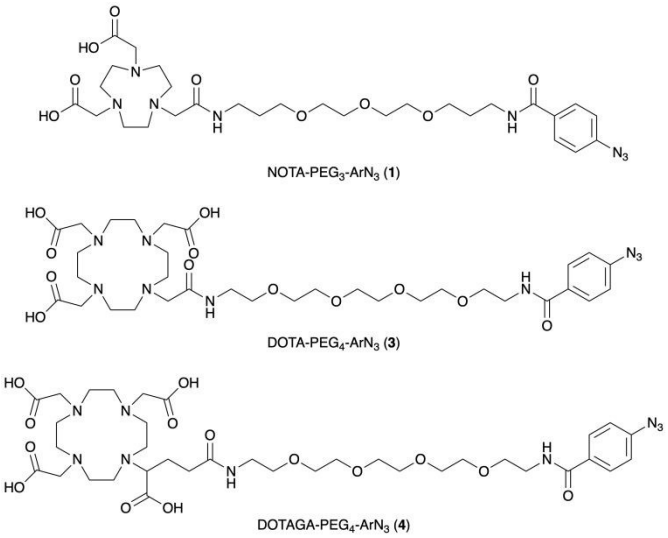

Figure 1. Chemical structures of photoactivatable macrocyclic chelates.

In addition to the chelates, the non-radioactive Ga complexes were produced and characterised by HR-ESI-MS and UHPLC (Figure 2, red trace, Figures S19 – S23). Radiolabelling experiments were monitored by radioactive instant thin layer chromatography (radio-iTLC) and radioactive UHPLC. Experiments showed that the chelates readily coordinated $^{68}Ga^{3+}$ ions (Figure 2, blue trace) and that the radioactive complexes co-eluted with the authenticated non-radioactive Ga-complexes as determined *via* comparison of the retention times (t_R / min), and also by standard co-injection methods. For compound **1**, formation of ^{68}Ga-**1**$^+$ was complete in <5 min. at room temperature. In contrast, synthesis of ^{68}Ga-**3** and ^{68}Ga-**4**$^-$, from the DOTA (**3**) and DOTAGA (**4**) derivatives, respectively, required heating to 70 °C for approximately 5 min. to affect complete complexation. A potentially useful feature of this set of chelates is that the varying number of carboxylate groups in compounds **1**, **3** and **4**, means that, under physiological conditions (pH7.4), the Ga^{3+} (and other 3+ metal ion) complexes will have different overall charges ranging from +1 to –1.

Following successful radiolabelling experiments on the chelates, the photochemical reactivity of the ^{68}Ga-complexes was tested. Radiochemically pure samples of ^{68}Ga-**1**$^+$, ^{68}Ga-**3** and ^{68}Ga-**4**$^-$ were irradiated using an intense light-emitting diode (LED, 365 nm, 10 – 30 min., room temperature). Subsequent radio-iTLC and radio-UHPLC analysis confirmed that the radioactive complexes reacted rapidly under UV irradiation to give essentially a single major new radioactive species (Figure 2, black trace). The photodegraded products each eluted with shorter retention times indicating that the new species are more hydrophilic than the parent complexes. Under the conditions employed, photoactivation of ArN_3 produces the short-lived arylnitrene species in the singlet ($^1A_2$) ground state.[20] When the *ortho*-positions with respect to the N atom are accessible, rapid intramolecular rearrangement of the singlet arylnitrene occurs to give a benzazirine species that undergoes ring expansion to yield a ketenimine intermediate.[21] In the absence of more powerful nucleophiles (primary or secondary amines), it has been shown that the ketenimines intermediate reacts with water to give the more polar azepin-2-ol species (or equivalent tautomers[22]) as the major photodegradation product. Experimental data on photochemical reactivity of ^{68}Ga-**1**$^+$, ^{68}Ga-**3** and ^{68}Ga-**4**$^-$ are consistent with this mechanism.

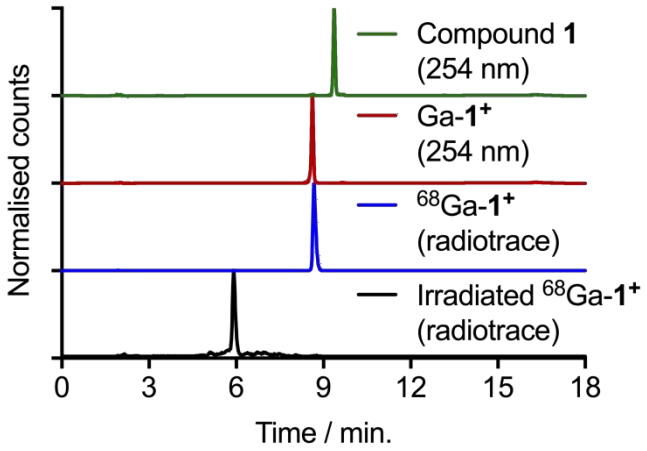

Figure 2. Normalised RP-UHPLC data showing, (green) a single peak for the elution of compound **1**, (red) a single peak observed for non-radioactive complex Ga-**1**$^+$, (blue) co-elution of ^{68}Ga-**1**$^+$ confirming the identity of the radioactive complex, and (black) a single peak formed after irradiation of ^{68}Ga-**1**$^+$ (365 nm, 15 min.). Equivalent data using compounds **3** and **4** are presented in the supporting information.

The two-step, one-pot photoradiochemical approach for radiolabelling trastuzumab, and structures of the three products is shown in Scheme 1. Standard $^{68}Ga^{3+}$ radiochemistry is not perfectly compatible with the photochemical conjugation step because the complexation reaction is performed under acidic conditions (pH~4.4, NaOAc buffer). In contrast, the photochemical conjugation proceeds most efficiently under slightly basic conditions where the nucleophilicity of the lysine side-chain is increased *via* deprotonation of the primary ε-NH_2 amine (pK_a ~10.5). For this reason, the chelates were pre-radiolabelled with $[^{68}Ga][Ga(H_2O)_6]^{3+}$ before adjusting the pH *in situ* to >7.5 using $NaHCO_3$ solution. Complex formation was monitored by radio-iTLC and radio-size-exclusion chromatography (SEC) UHPLC. After complete complexation, an aliquot of pre-purified trastuzumab was added with an initial chelate-to-mAb ratio of ~10-to-1. Reaction mixtures were then irradiated for 15 min. at room temperature. Aliquots of the crude reaction mixtures were analysed by radio-iTLC, manual

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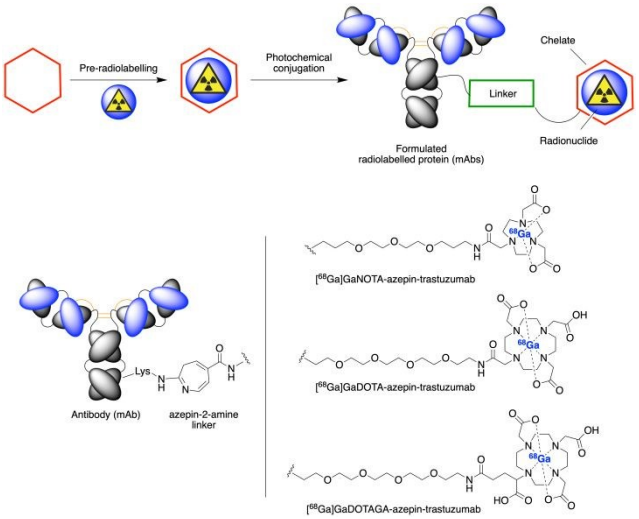

Scheme 1. (Top) Pre-radiolabelling and photochemical concept. (Bottom) Chemical structures of [^{68}Ga]GaNOTA-azepin-trastuzumab, [^{68}Ga]GaDOTA-azepin-trastuzumab and [^{68}Ga]GaDOTAGA-azepin-trastuzumab synthesised by two-step radiolabelling and photochemical conjugation.

size-exclusion chromatography (PD-10-SEC) and radio-SEC-UHPLC. In addition, a fraction was purified by preparative PD-10 and spin-centrifugation methods to measure the absolute radiochemical yield (RCY), radiochemical purity (RCP) and molar activities of the purified ^{68}Ga-radiolabelled trastuzumab (Figure 3 and Supporting Information Figures S24 and S25). Note, all experiments were performed in triplicate with independent replicates.

Starting from either compound **1**, **3** or **4**, ^{68}Ga-radiolabelled trastuzumab was produced in crude radiochemical yields of around 16 – 18%, as measured by analytical PD-10-SEC, and 11 – 16%, as measured by radioactive SEC-UHPLC (Figure 3). Based on the known initial concentrations of the reagents, the estimated final chelate-to-mAb ratios were in the range 1.1 to 1.8. For the radiochemical synthesis of [^{68}Ga]GaNOTA-azepin-trastuzumab, the purified sample was isolated in PBS with a decay-corrected RCY of 10.1 ± 0.7% (n = 3), a RCP >95%, and a molar activity, A_m of 0.46 ± 0.09 MBq nmol^{-1} of protein (n = 3; note, the protein concentration was remeasured after radioactive decay to obtain an accurate value). Our previous work found that photoradiochemical labelling does not compromise the biological activity (immunoreactivity) of the antibody.[18]

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In summary, experiments showed that photoactive derivatives of widely used *aza*-macrocyclic chelates (NOTA, DOTA and DOTAGA) are suitable for photochemical radiolabelling of proteins. Introduction of these photoactive chelates opens the possibility of using photoradiochemical methods with a broad range of radionuclides taken from across the periodic table. Ongoing work has also found that photoradiochemistry is a viable tool for synthesising radiolabelled peptides, small-molecules and nanoparticles, and that entire process can be automated (data to be reported elsewhere).

Conflicts of interest

There are no conflicts to declare.

Notes and references

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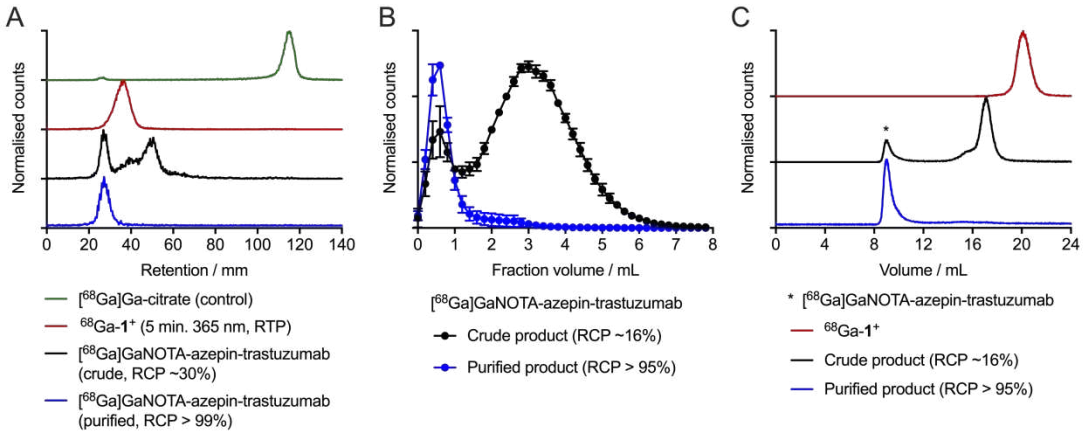

Figure 3. Characterisation data for the one-pot photoradiochemical synthesis of [^{68}Ga]GaNOTA-azepin-trastuzumab from pre-purified mAb. (A) Radio-iTLC chromatograms, (B) analytical PD-10-SEC elution profiles, and (C) SEC-UHPLC chromatograms of the crude and purified product. Equivalent data using compounds **3** and **4** are presented in the supporting information.

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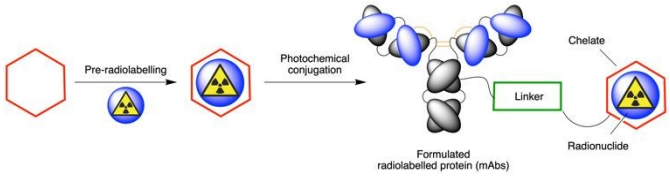

New photoactivatable ligands have been developed that facilitate one-pot photoradiochemical labelling of proteins with different radioactive metal ions.

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